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Effect of hydroalcoholic extracts of flower and fruit peel of *Punica granatum* on *Leishmania major* promastigotes *in vitro*

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Original Article

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Abstract:

Background and aims: Cutaneous leishmaniasis is one of the endemic parasitic diseases in many regions of Iran for which there is currently no complete vaccine and effective drugs without drug resistance to prevent and treat it. Given the need to develop new drugs for the treatment of various diseases with fewer side effects, greater effects with shorter treatment periods, especially in anti-leishmaniasis drug compounds for drug-resistant types of the disease, it is essential to do research on plant compounds containing medicinal compounds to promote the treatment of this infection. The present study was performed to compare the *in vitro* effects of hydroalcoholic extracts of flower and fruit peel of *Punica granatum* on *Leishmania major* promastigotes.

Methods: Different dilutions of hydroalcoholic extracts of flower and fruit peel of *P. granatum* were prepared by the maceration method and their *in vitro* effects on *L. major* promastigote were investigated using MTT assay. Data were analyzed using SPSS software version 22.

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Results: The results showed that both hydroalcoholic extracts of flower and fruit peel of *P. granatum* could inhibit the parasite, but the extract fruit peel of *P. granatum* showed anti-leishmaniasis activity at lower concentrations compared to extract flower of *P. granatum*.

Conclusion: The hydroalcoholic extracts of flower and fruit peel of *P. granatum* could inhibit the parasite, that probably due to the presence of some compounds such as ellagic acid with antioxidant properties and punicalagin with antimicrobial properties especially in fruit peel extract of *P. granatum*.

Keywords: *Punica granatum fruit peel, Punica granatum flower, Leishmania major, antiparasitic activity, in vitro techniques.*

INTRODUCTION

Leishmaniasis is a parasitic disease caused by various obligate intracellular flagellated protozoa of the genus *Leishmania*, which is transmitted by female nocturnally biting sand flies, *Phlebotomus* spp and *Lutzomia* spp in the Old World and New World respectively. Clinical manifestations of leishmaniasis can be cutaneous, diffuse cutaneous, mucocutaneous, and visceral. Cutaneous leishmaniasis causes skin lesions and is self-healing, mucocutaneous leishmaniasis leads to partial or total destruction of mucous membranes of the nose, mouth and throat and its visceral type, which is very fatal is known as visceral leishmaniasis or kala-azar. Identification the carriers of *leishmani* spp. and ways of transmitting infection along with control programs have not yet helped to completely control the disease so that it is still considered a public health problem by the World Health Organization (WHO) (1). According to the reports of WHO, leishmaniasis is endemic to 98 countries in the tropical and subtropical regions worldwide. According to epidemiological studies, the number of people with leishmaniasis is estimated at 12 million, and over 350 million people worldwide are at risk of developing it. Two million new cases of leishmaniasis are reported each year, with cutaneous leishmaniasis cases being approximately three times higher than visceral leishmaniasis (2). Currently, the drugs used to treatment leishmaniasis include glucantime, pentamidine isotianite, and amphotericin B, which have side effects such as recurrence of the disease with a long course of treatment, small effect,

renal and liver problems as well as drug resistance (3). Therefore, in recent years many studies have been conducted on the different effective compounds against leishmaniasis with fewer side effects, faster recovery, and affordability such as medicinal plants (4). Pomegranate tree (*Punica granatum*) belongs to the genus Myrtales and the family Punicaceae. This plant is a small tree or shrub, which is found in tropical and subtropical regions and has long been cultivated in some countries such as Iran, Egypt, India, the USA (California), Italy and China (5). Different parts of this plant such as tree bark, leaves, roots, flowers, peel of immature and mature fruits, seeds and juice have many biochemical compounds and different medicinal properties that are usually used in traditional medicine to treatmeant various diseases such as diarrhea, dysentery, tapeworm, parasitic infections of the urinary tract, bleeding, respiratory diseases and kidney stones (6). Pomegranate is a natural source of antioxidants such as tannins, polyphenols (ellagic acid, ellagic-gallic acid and coumaric acid), flavonoids (luteolin, kaempferol and naringin), vitamins (beta-carotene, ferrocyte, B2, C, B1), sugars (fructose, sucrose, and maltose), anthocyanins, fatty acids (linolenic acid, linoleic acid and oleic, palmitic, stearic, palmitoleic, arachidonic acid, caprylic fatty acids), aromatic compounds, amino acids, alkaloids, tocopherols, sterols, and terpenoids(7, 8).Therefore, different parts of this plant is used as a source of antimicrobial (9, 10), anti-parasitic (11, 12) anti-cancer and anti-inflammatory(13, 14), anti-mutation and antiviral (15) agents, and to prevent cardiovascular disease(16) and heal wounds (17) in different countries. The therapeutic properties of *P. granatum* fruit peel extracts are well known in the cultures of different countries. In Egyptian culture, several chronic diseases such as inflammation, diarrhea, intestinal worms, cough, and infertility were treated with *P. granatum* fruit peel extract (8). In addition, *P. granatum* flower that has a large structure (3.5-7 cm) and is reddish, cylindrical and odorless, occurs in fertile (large with l long stamen and style) and infertile (named golnar, small with short style and stamen) types, and contains estrone,

alkane, asiatic acid, maslinic acid, and ursolic acid, and cytosterol (18). Due to properties such as antibacterial, anti-inflammatory, anti-tumor, and antioxidant, *P. granatum* flower extract is used to treat acute ulcers, bronchitis, diarrhea and digestive problems, bleeding and diabetes, and to protect the liver (19). Given the confirmed therapeutic effects of the flower and fruit peel of *P. granatum* against bacterial and parasitic infections, we investigated the *in vitro* leishmanicidal effect of hydroalcoholic extracts of flower and fruit peel of *P. granatum* against *L. major* as pathogenic parasitic strain.

MATERIALS AND METHODS

Hydroalcholic extraction of flower and fruit peel of *P. granatum*

The flowers and fruits of *P. granatum* were purchased in the autumn of 2018 from the centers approved by Shahrekord University of Medical Sciences. Identification the flower species (herbarium code: 558) and fruit peel (herbarium code: 559) of *P. granatum* were done in Medicinal Plants Research Center of Shahrekord University of Medical Sciences. After being washed them with distilled water to remove dust and possible surface contamination, the samples of flower and fruit peel were dried in shade at room temperature (20-25 °C) for 3 and 8 days respectively. Extraction was done by the maceration method. By this method, the dried flowers and fruit peels of *P. granatum* were soaked in 50 ml of 80% ethyl alcohol (Merck, Germany) in a concentration with the ratio of 1:3 and shaken for 72 hours. Then, the supernatant was filtered through Whatman filter paper grade 1. The liquid obtained was concentrated using a rotary apparatus (Heidolph WD 2000, Brinkmann, Canada) at 50 °C and incubated at 37 °C for 3 days and stored at 4 °C until use (20).

Parasite culture

The standard strain of *L. major* (MRHO/75/ER) was procured in microtube which stored in -70 °C from the Department of Parasitology, School of Medicine, Isfahan University of Medical Sciences. After thawing, the strain was transferred to biphasic Novy Mack Neal Nicole (NNN) medium (Himedia, India) and incubated at 26°C. After the growth and proliferation of

promastigotes in the liquid phase and reaching the logarithmic phase, they were transferred to the Roswell Park Memorial Institute culture medium (RPMI 1640, Himedia India) enriched with 10% fetal bovine serum (FBS) (SIGMA, USA), whose complement had been previously inactivated at 56 °C for 30 minutes, containing penicillin antibiotics U/Ml100 (Himedia, India), streptomycin (U/Ml100) (Himedia, India). The culture medium was incubated for 4 days at 26 °C and the growth of parasites was monitored daily using an invert microscope. After the promastigotes reached the growth logarithmic phase, they were counted using a homocytometer slide and adjusted to 5×10^6 cell/ml for subsequent investigations (21).

Anti-leishmaniasis effects of flower and fruit peel of *P. granatum* using the MTT assay

The MTT assay is a colorimetric method in which tetrazolium salt is converted to an insoluble formazan dye solution. This regenerative reaction is mediated by the activity of the parasite mitochondrial succinate dehydrogenase, which is used as an indicator of the growth and viability of promastigotes against the drug response (22). To prepare 0.05% MTT solution, 5 mg of MTT powder (SIGMA, USA) was dissolved in 1 ml of sterile phosphate-buffered saline (PBS) solution (CinnaGen Co., Iran). From *L. major* promastigotes in stationary phase in RPMI-1640 medium, 100 µl was removed and added to each well of the plate so that 5×10^5 promastigotes were placed in each well. Then, the flower and fruit peel extracts of *P. granatum* were prepared at concentrations of 12.5, 25, 50, 100, 200, 300, 400, and 500 mg/ml and 10 µl of each was added to each well of 96-well plate (23). Also, A well containing 10 µg/ml glucantime was considered as positive control , a well containing untreated the parasite promastigotes as negative control, and a well containing the RPMI culture medium containing 10% parasite-free FBS as blank. Then, 10 µl of the MTT solution at a concentration of 5 mg/ml was added to each well at different intervals (0, 6, 24, 48 and 72 hours after treatment) (24). The plates were covered with aluminum foil and incubated at 26 °C for 3-4 hours, and then a volume equivalent to the initial

culture medium of 100 µl of dimethyl sulfoxide (DMSO) solution (CinnaGen Co., Iran) was added to each well and gently mixed of medium and cells, so that there were no sediment seeds and the formed formazan crystals were dark purple. Finally, the optical absorbance of each well was read using ELISA reader at 570 nm with reference to a 630-nm filter. All the procedures were done in triplicate and then IC50 (Inhibitory concentrations 50%), i.e., the concentration of the extract that prevents the growth of 50% of the organism, was calculated. To this end, the percentage of parasite survival was calculated using the following formula:

The percentage of parasite survival = [(AT-AB)/AC-AB)]×100

In this formula AT is optical absorption of cells which treat with different concentration of flower and fruit peel extracts of *Punica granatum* and glucantime , AC= optical absorption of the untreat cells (control) , and AB= optical absorption of the sample without cell of RPMI (blank OD) (25).

IC_{50} of different concentrations and times were calculated by Microsoft Excel and were compared with SPSS software version 22.

Log (IC50) = log(x1)+[y1-y0/2)/(y1-y2)] [log(x2)-log(x1)].

Statistical Analysis

All assays were repeated three times and the results were expressed as the mean ± standard deviation. All data were compared by analysis of variance (One-Way ANOVA). Moreover, to compare the IC50 values of the groups, t-test was performed difference was considered significant when $p<0.05$.

RESULTS

In this experimental study, the *in vitro* effects of different concentrations of the flower and fruit peel extracts of *P. granatum* on *L. major* promastigotes were studied based on F90 (90% fatality) and IC50 using the MTT assay. The IC50 of fruit peel extract of *P. granatum* at intervals of 0, 6, 24, 48 and 72 hours were obtained 191.34, 180.99, 152.11, 129.37 and89.76 µg/ml, and this rate for flower extract of *P. granatum* at intervals of 0, 6, 24, 48 and 72 hours were 375.51, 340.49, 293.23, 211.96 and 125.12 µg/ml, respectively. The F90 of fruit peel extract of *P. granatum* at intervals of 0, 6, 24, 48 and 72 hours were obtained 715.29, 712.32, 664.28, 691.50, and 626.08 mg, and for flower extract of *P. granatum* were 1283.89,

1310.40, 1412.76, 1560.84, and 1234.41 mg, respectively (Tables 1 and 2).

Table 3 shows the mean optical absorbance of the studied concentrations of hydroalcoholic extract of fruit peel of *P. granatum* by time in MTT assay.

Table 1: The inhibitory concentration of 50% and 90% fatality of hydroalcoholic extract of fruit peel of *P. granatum* on *L. major in vitro*.

extract	Time (h)	Ic50 (mg/ml)	confidence interval 95%	F90	confidence interval95%
fruit peel of *Punica granatum*	0	191.34	173.19 & 209.45	715.29	661.99 & 780.69
	6	180.99	164.56 & 197.22	712.32	663.98 & 770.45
	24	152.11	131.76 & 171.49	664.28	611.69 & 729.75
	48	129.37	112.70 & 145.09	691.50	646.90 & 744.56
	72	89.76	62.32 & 113.81	626.08	569.45 & 699.05

Table 2: The inhibitory concentration of 50% and 90% fatality of hydroalcoholic extract of flower of *P.granatum* on *L. major in vitro*.

extract	Time (h)	Ic50 (mg/ml)	confidence interval 95%	F90	confidence interval95%
flower of *Punica granatum*	0	375.51	354.66 & 399.21	1283.89	1192.61 & 1393.19
	6	340.49	313.11 & 372.89	1310.40	1178.09 & 1482.94
	24	293.23	277.89 & 309.82	1412.76	1320.72 & 1520.87
	48	211.96	195.19 & 229.0	1560.84	1438.65 & 1709.21
	72	125.12	109.65 & 139.67	1234.41	1155.44 & 1327.09

There was no significant difference in optical absorbance between 12.5, 25 and 50 mg concentrations at all intervals, but there was a significant difference at other concentrations (P<0.001). At 24h interval, there was no significant difference in optical absorbance between 12.5 and 25 mg concentrations while there was a significant difference at other concentrations (P<0.001). At 48h interval, there was no significant difference in optical absorbance between 12.5 and 25, and 400 and 500 mg concentrations, but there was a significant difference at other concentrations (P<0.001, Figure 1).

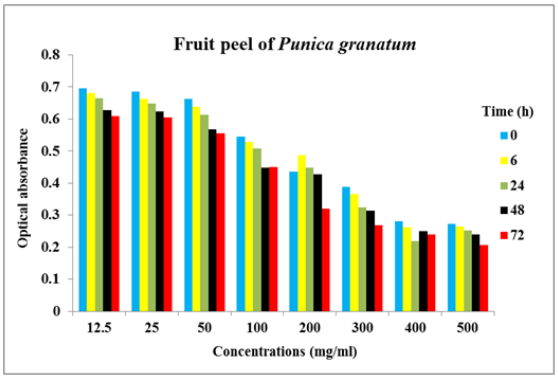

Figure 1. Mean optical absorbance of the studied concentrations of hydroalcoholic extract of fruit peel of *Punica granatum* by different times in MTT assay.

Table 4 shows the mean optical absorbance of the studied concentrations of flower extract of *P. granatum* by different times. At 0-h interval, concentrations of 25 and 50 mg and concentrations of 400 and 500 mg were not significantly different in terms of optical absorbance, but there was a significant difference at other concentrations (P<0.001).

At 6h interval, there was no significant difference in optical absorbance between concentrations of 25 and 12.5, and 25 and 50 mg. Concentrations of 300 and 400, and 300 and 500 mg, and 400 and 500 mg were not also significantly different in optical absorbance.

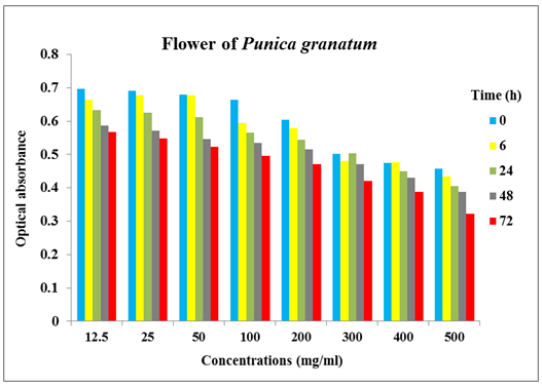

Figure 2. Mean optical absorbance of the studied concentrations of hydroalcoholic extract of flower of *Punica granatum* by different times in MTT assay.

At 24h interval, there was no significant difference in optical absorbance between concentrations of 25 and 12.5, and 25 and 50 mg. In addition, concentrations of 100 and 200 mg were not significantly different in terms of optical absorbance but there was a significant difference in optical absorbance at other concentrations (P<0.001).

At 48h interval, there was no significant difference in optical absorbance between concentrations of 12. 5 and 25, 100 and 50, and 100 and 200 mg. At 72h interval, optical absorbance at 25mg concentration was not significantly different from that at 12.5 and 50mg concentrations, but there was a significant difference in optical absorbance at other concentrations (P<0.001, Figure 2).

Table 3. Mean optical absorbance of the studied concentrations of hydroalcoholic extract of fruit peel of *Punica granatum* by different timesin MTT assay.

Time(h) / Concentration(mg)	0	6	24	48	72
12.5	0.695±0.003	0.682±0.004	0.664±0.005	0.627±0.005	0.608±0.006
25	0.685±0.004	0.663±0.004	0.648±0.004	0.623±0.004	0.605±0.011
50	0.662±0.002	0.683±0.009	0.614±0.014	0.567±0.012	0.555±0.007
100	0.545±0.013	0.528±0.005	0.507±0.006	0.499±0.007	0.451±0.012
200	0.436±0.010	0.487±0.005	0.449±0.009	0.427±0.026	0.321±0.018
300	0.388±0.003	0.366±0.008	0.325±0.014	0.314±0.015	0.269±0.014
400	0.280±0.011	0.262±0.005	0.218±0.006	0.250±0.002	0.239±0.010
500	0.272±0.017	0.265±0.025	0.253±0.007	0.239±0.008	0.207±0.006

Table 4: Comparison of mean optical absorbance of the studied concentrations of hydroalcoholic extract of flower *Punica granatum* by different times in the MTT assay.

Time(h) / Concentration(mg)	0	6	24	48	72
12.5	0.696±0.005	0.663±0.027	0.632±0.017	0.587±0.007	0.568±0.009
25	0.691±0.001	0.677±0.008	0.626±0.009	0.571±0.009	0.547±0.008
50	0.679±0.007	0.678±0.018	0.612±0.006	0.546±0.007	0.523±0.008
100	0.664±0.006	0.594±0.009	0.566±0.007	0.534±0.012	0.496±0.004
200	0.604±0.011	0.578±0.011	0.544±0.009	0.516±0.005	0.470±0.011
300	0.502±0.009	0.481±0.027	0.504±0.11	0.471±0.008	0.421±0.009
400	0.475±0.006	0.477±0.008	0.450±0.011	0.430±0.008	0.388±0.008
500	0.458±0.010	0.435±0.006	0.406±0.007	0.387±0.006	0.323±0.014

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Discussion

According to the WHO, leishmaniasis is one of the six leading infectious parasitic diseases worldwide. The chemical drugs used to treat leishmaniasis include pentavalen antimonial compounds such as Pentostam, glucantime and amphotericin B. Due to the innate resistance of some strains of *leishmania* spp to drugs, toxicity and side effects of chemical drugs, also disease recurrence, high cost and long treatment, the use of medicinal plants for treating leishmaniasis has recently received much attention (3, 4). In this study, the *in vitro* effects of hydroalcoholic extracts of flower and fruit peel of *P. granatum* were comparatively investigated on *L. major* promastigotes. Many Studies have indicated the presence of different compounds with antimicrobial properties in plants. The fruit peel of *P. granatum* contains phenolic compounds such as ellagic acid, ellagitannins, gallic acid, anthocyanin and pyridine alkaloids, also flowers of *P. granatum* contain compounds such as asiatic acid, gallic acid, ursolic acid, and terpenoids as well as phenolic compounds such as

punicalagin, therefore these components have antimicrobial and antifungal effects (26). Minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) are used to investigate the antimicrobial activity of synthetic and organic compounds extracted from plants. In the present study, the results on IC50 and F90 in probit test for *L. major* showed that with increasing the exposure time of the extract to the parasite, the effect of the extract on the parasite and fatality rate increased. The IC50 fruit peel extract of *P. granatum* at intervals of 0, 6 and 24 hours were obtained 191.34, 180.99 and 152.11 µg/ml, and flower extract of *P. granatum* were 375.51, 340.49, and 293.23 µg/ml, respectively. So, both flower and fruit peel extracts of *P. granatum* could inhibit the proliferation of parasite. However, in fact fruit peel extract of *P. granatum* had anti-leishmaniasis activity at lower concentrations than extract flower of *P. granatum*, which is probably due to the presence of some compounds such as ellagic acid and punicalagin in fruit peel extract of *P. granatum*. Ellagic acid is the most important phenolic compound in fruit peel of *P. granatum* and has a phenolic structure and antioxidant properties. Besides that, punicalagin exhibits antimicrobial and antifungal properties(26). In a study conducted by Haddad et al., the antiparasitic *in vitro* effect of hydroalcoholic extract fruit of *P. granatum* on *L. major* was investigated, but the effect of flower extract of *P. granatum* on this parasite was not investigated. In that study, the *in vitro* effect of fruit peel extract of *P. granatum* at concentrations of 15.12, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml on *L. major* was investigated. The IC50 in that study was obtained 138 µg/ml, but in the present study, IC50 was 89.76 µg/ml at 72 hours and the extract inhibited the proliferation of parasite at a lower concentration compared to the findings of Haddad et al. In addition to its anti-inflammatory and antioxidant properties, fruit peel of *P. granatum* has anti-parasitic properties due to the presence of nitazoxanide in its compounds. According to the study of Haddad et al. the greatest inhibitory effect on the growth of promastigotes was observed at 500-1000 µg/ml at 72h interval and other concentrations had no anti-leishmania effect (27), but in the present

study all concentrations had an anti-*leishmania* effect and with increasing exposure time, the anti-*leishmania* effect of the extract increased. Because in the study of Haddad et al., extraction was performed with 70% methanol solvent and different solvents cause differences in the concentration and type of compounds extracted from the plant, the inconsistency in the results may be due to differences in the method of extraction, genotypic differences and environmental factors affecting the cultivation of the plants used in the studies. Numerous studies have been performed on the *in vitro* and *in vivo* effects of fruit peel and flower extracts of *P. granatum* on parasites and the antimicrobial effect of the extract, all of these components exhibited promising effects. In this regard, Alkathiri et al. compared the effects of fruit of *P. granatum* on *L. major* at concentrations of 10-200 µl/ml *in vitro* and at concentrations of 0.8 µl/ml *in vivo* administered orally for four week in mice compared to Pantostam (120 mg/kg subcutaneous injection). The results of this study showed that *in vitro*, 200 µl/ml of *P. granatum* fruit juice caused the death of more than 83.7% promastigotes of *L. major* and the IC50 of *P. granatum* fruit juice was 118.2 µl/ml. Also, The treatment of wounds induced by inoculated *L.* major in mice showed a strong effect of *P. granatum* fruit juice in comparison with Pentostam, so that the juice completely healed wounds due to the presence of phenolic and flavonoid compounds, especially luteolin, ellagitannin and epigallocatechin,As well as, the anti-inflammatory and antioxidant effects of *P. granatum* fruit juice are produced by activating the intra-macrophage nitric oxide and inducing interferon-gamma cytokines tumor necrosis factor in mice (28). In another study, Imperatori et al. investigated the effect of fruit peel of *P. granatum* at concentrations of 10-275 µg/ml on *L. infantum* promastigotes *in vitro* using direct microscopical, DNA degradation and electron microscopical methods. Their results showed that hydroalcoholic extract fruit peel of *P. granatum* caused the parasite apoptosis and death them due to the presence of tannins in the fruit peel (29). In other studies, the antiparasitic properties of leaf and fruit extracts of *P. granatum* against promastigotes and amastigotes of *L. amazonensis* and amastigotes of *L.*

infantum have been investigated, which indicate the anti-*leishmania* properties of various compounds of *P. granatum* (30, 31). Ahmadi et al. examined the antimicrobial activity of hydroalcoholic extract flower of *P. granatum* obtained by ethanol maceration on *Escherichia coli* and *Staphylococcus aureus*. According to their results, the MIC for both bacteria was 7.8 μg/ml and both bacteria showed high sensitivity to flower extract *P. granatum* (26). Phytochemical investigations performed on different extracts of *P. granatum* confirmed the presence of phenolic compounds in the extract samples. Ahmadi et al. studied total phenolic content in hydroalcoholic extract flower of *P. granatum*, and their results showed that the antibacterial activity flower extract of *P. granatum* was directly related to total phenolic content, which is 133.15 mg gallic acid equivalent/g of the sample (26). In the present study, the inhibitory effect flower extract of *P. granatum* on *L. major* promastigotes was probably due to the presence of these phenolic compounds. Rakhshandehroo et al. investigated the effect of methanolic flower extract of *P. granatum* on larvae of *Parascaris equorum* at concentrations of 50, 75, 100 and 125 mg/ml at 0, 10, 20, 30 and 40-minute intervals *in vivo*, and observed that the extract at all concentrations inhibited intestinal worms (32). In this study, there was a significant relationship between the concentration and the interval time of exposure to the extract, and the fatal activity of the extract. The IC50 flower extract of *P. granatum* at 0, 6h and 24h interval times were 375.51, 340.49, and 293.23 respectivelly, so that increasing exposure time and concentration of extracts increased the inhibitory impact of the extract on the parasite (32), which is consistent with the present study. In the present study, the alcoholic extracts of flower and fruit peel of *P. granatum* exhibited a high anti-*leishmania* effect. Some studies have shown that alcoholic extract has a higher antimicrobial and anti-leishmania effect than aqueous extract, which is probably due to the extraction of higher amounts of antioxidants and phenolic compounds from alcoholic extracts than from aqueous extract of plants.

CONCLUSION

The results of the present study showed that given the F90 and IC50 of *L. major*, the flower and extract fruit peel of *P. granatum* had a significant effect on inhibiting the parasite *in vitro*.

Authors' contributions

Bahman Khalili and Rahman Abdizadeh contributed to the design and supervision of the study, Hamidreza Mardani, Sedigheh Saberi and Zahra Lorigooini contributed to the laboratory procedures. Milad Ghaderi, Rahman Abdizadeh and Hamidreza Mardani contributed to drafting and reviewing the article.

CONFLICT OF INTERESTS

Authors declare no conflicts of interest.

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